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In vitro comparison of duration of action of melatonin agonists on melatonin MT_1 receptor: Possible link between duration of action and dissociation rate from receptor

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ABSTRACT

Melatonin MT_1 and MT_2 receptors are Gi protein-coupled receptors and promising therapeutic targets for a number of diseases. A proportion of G protein-coupled receptor agonists and antagonists have been classified according to their duration of action, which influences their pharmacological efficacy. However, the duration of action of melatonin agonists remains unclear. In this study, we investigated the duration of action of melatonin agonists (melatonin, 2-iodomelatonin, ramelteon, and the ramelteon metabolite M-II) at the melatonin MT_1 receptor, which is more resistant to agonist-induced desensitization than the melatonin MT_2 receptor. In Chinese hamster ovary cells stably expressing the human melatonin MT_1 receptor, significant differences in the duration of action were observed after 2-h pretreatment with agonists followed by washout. In contrast to melatonin and M-II, the agonist activities of ramelteon and 2-iodomelatonin were persistent (*i.e.* inhibition of forskolin-stimulated cAMP formation and increase in ERK 1/2 phosphorylation) even after repeated washouts. Similar activities were observed for INS-1 cells endogenously expressing the rat MT_1 receptor. Further, we examined potential factors linked to the duration of action. Residual activities of melatonin agonists after washout strongly correlated with their dissociation rates from the human melatonin MT_1 receptor, but not their lipophilicity or extent of desensitization. These data suggest that the *in vitro* duration of action significantly differs between melatonin agonists and might dictate dissociation kinetics. Characterization of these *in vitro* properties may facilitate further *in vivo* study of the duration of action.

1. Introduction

Melatonin is a circulating hormone primarily secreted by the pineal gland during the night in diurnal and nocturnal animals, which has been implicated in sleep-wake cycles, circadian entrainment, and seasonal reproduction (Dijk and Cajochen, 1997; Redman et al., 1983; Reiter, 1980). These effects are mediated *via* the two distinct G protein-coupled receptors MT_1 and MT_2, which suppress cAMP production *via* a Gi protein (Reppert et al., 1994, 1995). Both receptors are expressed in various areas of the central nervous system and peripheral tissues; notably, the melatonin MT_1 receptor is highly expressed in the human suprachiasmatic nucleus (SCN) (Dubocovich et al., 2010; Hardeland, 2012). This brain structure is considered the major site in which melatonin exerts phase-shifting and sleep-inducing effects (Hardeland, 2012). Several melatonin agonists have been developed and tested in humans, including ramelteon for insomnia and agomelatine for depression (Erman et al., 2006; Kennedy and Emsley, 2006).

A proportion of G protein-coupled receptor agonists and antagonists have been classified by their duration of action. For example, clinically available β_2-adrenoceptor agonists vary in terms of duration of action, with salbutamol being a short-acting agonist requiring administration several times a day and indacaterol being a long-acting agonist requiring administration once a day (Battram et al., 2006; Waldeck, 2002). In addition to pharmacokinetics, other factors influencing the duration of action of β_2 adrenoceptor agonists have been reported (Baur et al., 2010; Coleman et al., 1996; Deyrup et al., 1999; Szczuka et al., 2009). Thus, the characterization of the duration of action and related factors of melatonin agonists would be required to elucidate their pharmacological efficacy. Further, the duration of action might reflect receptor desensitization.

Several studies have examined desensitization of melatonin receptors *in vitro* and *in vivo*. Melatonin treatment of Chinese

Abbreviations: CHO, Chinese hamster ovary; CHO-hMT_1, CHO cells stably expressing human melatonin MT_1 receptor; FBS, fetal bovine serum; mHBSS, modified Hanks' balanced salt solution; PTX, pertussis toxin; PBS, phosphate-buffered saline; SCN, suprachiasmatic nucleus

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hamster ovary (CHO) cells expressing the human melatonin MT_1 or MT_2 receptor induces more significant desensitization of melatonin MT_2 receptors than of MT_1 receptors (Gerdin et al., 2003). The prevalence of desensitization of melatonin MT_2 receptors has also been reported in SCN2.2 cells expressing endogenous melatonin MT_2 receptors (Gerdin et al., 2004b). Further, chronic melatonin treatment in Syrian hamsters that express melatonin MT_1 but not MT_2 receptors did not alter the sensitivity of SCN cells (Ying et al., 1998). Taken together, these previous findings suggest that, after treatment with pharmacological doses of melatonin, the function of the melatonin MT_1 receptor might be maintained due to its higher resistance to desensitization than the melatonin MT_2 receptor. Some studies have also examined the duration of action of melatonin on the melatonin MT_1 receptor (Gerdin et al., 2004a, 2004b; MacKenzie et al., 2002; Witt-Enderby and Dubocovich, 1996). However, little is known about the differences in the duration of action between melatonin agonists.

Here, the duration of action of melatonin agonists at the melatonin MT_1 receptor was investigated. Agonist activities were monitored to assess their functional duration of action in CHO cells stably expressing human melatonin MT_1 receptor (CHO-hMT_1), and potential factors linked to the duration of action were examined.

2. Materials and methods

2.1. Reagents

Chemical structures of all 7 melatonin agonists are presented in Fig. 1. Five melatonin agonists were synthesized at Takeda Pharmaceutical Company, Ltd. (Osaka, Japan), as follows: Ramelteon ((S)-N-[2-(1,6,7,8-tetrahydro-2H-indeno-[5,4-b]furan-8-yl)ethyl]propionamide) (Uchikawa et al., 2002), its monohydroxylated metabolite M-II ((2*S*)-2-hydroxy-N-[2-[(8*S*)-1,6,7,8-tetrahydro-2H-indeno[5,4-b]furan-8-yl]ethyl]propionamide), compound 1 ((1R,2R)-N-{[2-(2-methyl-2H-indazol-4-yl)cyclopropyl] methyl}propanamide) [WO Patent WO2008136382], compound 2 ((1R,2R)-N-{[2-(2-methylpyrazolo[1,5-a]pyridin-4-yl)cyclopropyl] methyl}cyclopropanecarboxamide) [WO Patent WO2008136382], and compound 3 ((S)-N-[2-(2-methyl-2,6,7,8-tetrahydrocyclopenta[e]indazol-8-yl)ethyl]acetamide) [WO Patent WO2008084-717]. Melatonin, pertussis toxin (PTX), 3-isobutyl-1-methylxanthine, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Iodomelatonin and luzindole were obtained from Tocris Cookson Ltd. (Bristol, UK), and 2-[^{125}I] iodomelatonin from PerkinElmer Inc. (Waltham, MA, USA). Anti-ERK 1/2 (#9107), phospho-ERK 1/2 (#9101), CREB (#9104), and phospho-CREB antibodies (#9198) were purchased from Cell Signaling Technology (Danvers, MA, USA). IRDye800CW-conjugated anti-rabbit IgG was obtained from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA). All other reagents were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2. Cell culture

CHO-hMT_1 cells were used as previously described (Kato et al., 2005). Cells were maintained in Eagle's minimum essential medium-α supplemented with 10% dialyzed fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin under an atmosphere of 5% CO_2/95% air. INS-1 832/13 cells were derived from INS-1 rat insulinoma cells and were provided by Dr. Christopher B. Newgard (Duke University Medical Center, Durham, NC, USA) (Hohmeier et al., 2000). INS-1 832/13 cells were maintained under a 5% CO_2/95% air atmosphere at 37 °C in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 10 mM HEPES, 10% heat-inactivated FBS, 55 μM 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.3. Membrane preparation

CHO-hMT_1 and INS-1 cells were washed with PBS and detached from plates using PBS containing 1 mM EDTA. Cells were then pelleted by centrifugation at 1000 × *g* for 5 min, resuspended in ice-cold 50 mM Tris–HCl buffer (pH 7.5–7.7), and stored at −80 °C until use. Cell membranes were prepared in 50 mM Tris–HCl buffer (pH 7.5–7.7) by homogenization and centrifugation at 40,000 × *g* for 20 min at 4 °C. This procedure was repeated before resuspending the final pellet in 50 mM Tris–HCl buffer (pH 7.5–7.7). Protein concentrations were determined using the Bicinchoninic Acid Protein Assay Kit.

2.4. Determination of association and dissociation rates of 2-[^{125}I] iodomelatonin

Binding reactions were initiated by the addition of 2 different concentrations of 2-[^{125}I] iodomelatonin (50 and 200 pM) to CHO membranes (approximately 30 μg of protein/assay tube) in 50 mM Tris–HCl buffer (pH 7.7) containing vehicle (dimethyl sulfoxide) or melatonin (10 μM). After incubation for 5, 10, 20, 40, 80, or 160 min at 37 °C, the reaction was terminated by the addition of ice-cold 50 mM Tris–HCl buffer (pH 7.7), followed by vacuum filtration through a Whatman GF/B filter. The filter was washed 3 times, and radioactivity was measured using a γ-counter (Hitachi Aloka Medical, Tokyo, Japan). Nonspecific binding was determined in the presence of 10 μM melatonin in all radioligand experiments.

2.5. Determination of dissociation rates of unlabeled melatonin agonists

CHO membranes were incubated in 50 mM Tris–HCl buffer (pH 7.7) containing 10 nM of each unlabeled ligand (melatonin, 2-iodomelatonin,

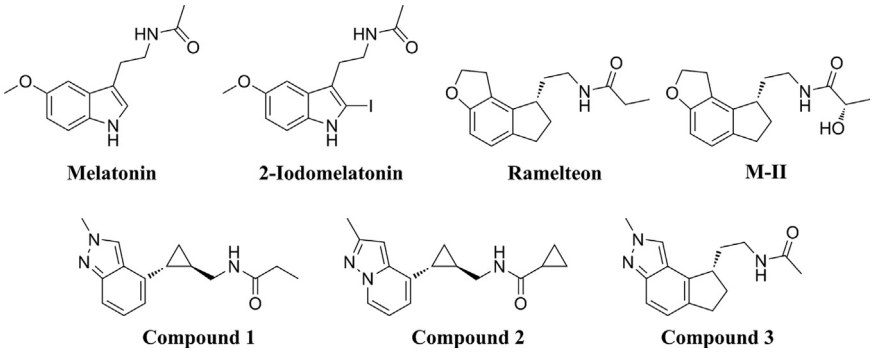

Fig. 1. Chemical structures of the tested melatonin agonists.